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In Vitro Lymphocyte Stimulation in an Approach to Study the Expression of HLA-encoded Genes Predisposing to Tuberculoid Leprosy

TO THE EDITOR:

Since the original observations that murine major histocompatibility complex located genes control susceptibility to Gross leukemia virus ([5]) and regulate—Ir genes—the immune response to synthetic polypeptides ([6]), a search for relationships between HLA and infectious diseases in man was made.

Especially in leprosy such a search appeared to be rewarding. The existence of HLA-encoded genes, contributing to the susceptibility to tuberculoid leprosy, was shown as well by means of population studies, as by means of family studies ([3]). The latter studies showed a recessive mode of inheritance of the susceptibility trait. Although the involvement of HLA-encoded Ir genes is supposed to underlie the observations made, no formal proof of this supposition has been obtained as yet.

One of the most reliable test systems currently used to study the expression of Ir genes *in vitro* is the lymphocyte transformation test (LTT) ([1, 4, 8]). The LTT measures the proliferative response of mononuclear cells, obtained from peripheral blood, in the presence of antigens. Healthy contacts of leprosy patients are known to show variability in this proliferative response after stimulation with *Mycobacterium leprae* antigens ([7]). Since clinical observations indicate that an *in vitro* proliferative response to *M. leprae* correlates with a state of hypersensitivity to *M. leprae,* it could well be that the individual variability in the LTT reflects the differential expression of HLA-encoded genetic factors, previously described to predispose to tuberculoid leprosy.

To test the above-mentioned hypothesis, we tested in the LTT healthy siblings (sibs) of tuberculoid leprosy patients who were either fully HLA-identical with their tuberculoid leprosy-affected sibs or HLA nonidentical with their tuberculoid sibs. This grouping was based on the recessive mode of inheritance, reasoning that only the fully HLA-identical sibs would carry the genetic make-up predisposing to tuberculoid leprosy. All individuals tested were derived from multicase tuberculoid leprosy families which were collected in Whardha District, Maharashtra, India, in collaboration with Dr. N. K. Mehra (AIIMS, New Delhi).

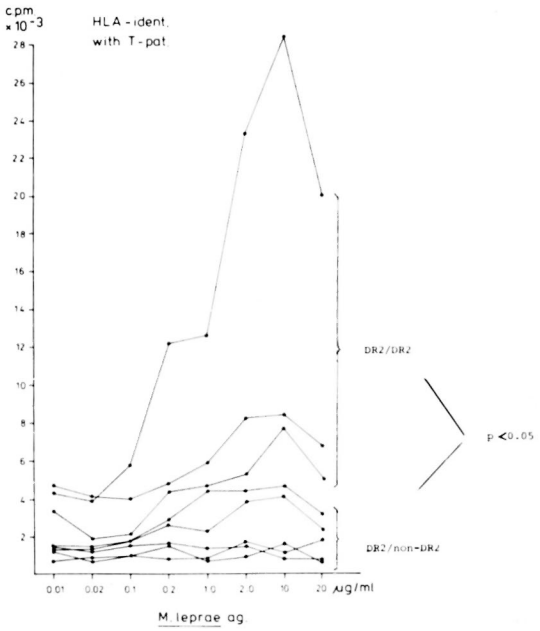
The segregation pattern of parental HLA-haplotypes has been reported previously ([9]). The mononuclear cells used for LTT were isolated on a Ficoll-Isopaque density gradient and successfully frozen in a freezing medium containing dimethylsulfoxide (10% in culture medium). For the freezing procedure a polystyrene box, containing 1 ml plastic ampules filled with the cells in freez-

ing medium, was placed in dry ice. In this way a cooling rate of approximately 1°C per min was obtained. The ampules were transported to Leiden, The Netherlands, on dry ice and stored in liquid nitrogen until used.

For the LTT, cells were thawed and resuspended to a concentration of 3×10^6 viable cells per ml in RPMI-1640 medium, containing inactivated pooled human serum. A standard LTT was carried out in flat-bottomed, 96-well microtiter plates with 0.1 ml of cell suspension and 0.1 ml of antigen suspension at various concentrations. All cultures were carried out in triplicate. Cultures were maintained for five days after which period 2 μCi of ^{3}H-thymidine was added for an additional 15 hr. Triplicate samples were harvested on glass filters and counted in a liquid scintillation counter. The cultures were stimulated with Dharmendra lepromin, kindly supplied by Dr. M. Abe (National Institute for Leprosy Research, Tokyo, Japan), in eight concentrations varying from 0.01 mg/ml to 20 mg/ml.

As expected, different degrees of *M. leprae*-specific lymphocyte reactivity were observed among the sibling contacts tested. However the individual degree of reactivity did not correlate with the fact of whether the individual tested was HLA-identical with the patient-sibling or not. These findings, therefore, do not support our working hypothesis and could be interpreted to disprove its tenability. Nevertheless, some facts point to a more limited conclusion. Firstly, the numbers of individuals tested, eight individuals being HLA-identical and another 12 individuals being HLA non-identical with their diseased contact sibling, were limited. Secondly, it could well be that the genetic constellation of the families under study is unfortunately too complex to detect the expression of the HLA-encoded genetic factors we are looking for. As described previously ([9]), in the sample of families from which the tested sib-pairs were derived, non-random allocation of the parental haplotypes among the patients was only detected by analyzing the HLA-DR2 positive haplotypes in particular.

The latter observation confirmed the previously reported association of HLA-DR2 with tuberculoid leprosy in India ([2]). If we take this into account, however, one observation might indeed support our working



THE FIGURE. LTT results with Abe lepromin of lymphocytes from healthy HLA-identical siblings of patients with TT leprosy.

hypothesis: Among the eight individuals, who are HLA-identical to their diseased sibs, three individuals do carry two HLA-DR2 positive haplotypes, which they share with their diseased HLA-DR2 homozygous sibs. As shown in The Figure, these three HLA-DR2 homozygous healthy contacts do show, each individually, a higher proliferative *M. leprae*-specific response, at each antigen concentration, than the other five ($p < 0.05$).

We are cautious in interpreting this remarkable finding, since the response of these three individuals does not rise significantly above the responses seen among the other group of individuals who are not fully HLA-identical to the patients. Further studies, along these lines, should be carried out to see whether the expression of HLA-encoded factors controlling leprosy may be detected in the LTT.

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Reduced Estrogen Excretion Due to Clofazimine?

TO THE EDITOR:

Biochemical monitoring of complicated pregnancies may be disturbed by the effects of medication taken by the mother. For example, urinary estrogen excretion, which provides a valuable index of feto-placental function and an accurate indicator of the risk of impending fetal death from placental insufficiency ([9]), is diminished by high-dose corticosteroid therapy ([8]) or by ampicillin ([10]). In a recent study of estrogen excretion in pregnant Ethiopian women with leprosy ([3]), we have been able to examine the impact of dapsone and clofazimine therapy on urinary estrogen values.

Dapsone treatment had little effect on estrogen excretion since there was no significant difference between the mean estrogen excretion in 13 women with "cured" tuberculoid leprosy and in seven women with active tuberculoid leprosy who received dapsone (50 mg–100 mg daily) (The Figure). Nevertheless, in women with lepromatous leprosy receiving dapsone alone, 52% of estrogen assays were subnormal.

Estrogen levels were further reduced in patients receiving clofazimine. The effect of immunosuppressive doses of clofazimine (300 mg daily) was studied in one patient who was already receiving prednisolone but whose initial estrogen excretion was within the normal range. Introduction of clofazimine therapy was associated with diminished estrogen excretion, although a live surviving infant (2.8 kg) was delivered at 39+ weeks of gestation. In three other women, who were already established on clofazimine (300 mg per week) for dapsone-resistant leprosy before estrogen assays were commenced, five out of six values (85%) were subnormal (relative to the lower limit of excretion in normal European women for the same period of gestation) ([7]).

In considering the possible risks of clofazimine to pregnancy, it should be borne in mind that most patients receiving clofazimine have lepromatous leprosy in reaction. Even in hitherto uncomplicated lepromatous leprosy, the risks of pregnancy are considerable; namely relapse ([2, 6]), emergence of dapsone resistance ([5]), reaction ([6]), and new nerve damage ([4]). Furthermore, with the increasing emergence of dapsone-resistant leprosy urgently requiring for its control introduction of dual- and triple-drug regimens ([1, 11]), it is likely that more women of childbearing age will receive clofazimine.

These findings suggest that there is a need